

***The MADS box of serum response factor is sufficient for
induction of muscle spindle-specifying gene Egr3 by
neuregulin***

An Honors Thesis (HONR 499)

By

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Abstract

Muscle spindles are an essential part of the neuro-muscular system because they act as sensory receptors of muscle and limb position that communicate with the nervous system and sense movement. It has been established that the neuregulin1 (NRG1)– ErbB signaling pathway plays a key role in the formation of muscle spindles. Our research aims to further characterize this pathway and more specifically the role of the transcription factor serum response factor (SRF), which previous evidence suggests may be a downstream effector during NRG1 signaling. To address how SRF is targeted during neuregulin signaling, we have generated muscle cells that inactivate endogenous SRF and express a truncated mutant form of SRF, using retroviral transduction and CRISPR/Cas9. These cells were then stimulated with NRG1 and qRT-PCR was used to measure expression of Early growth response 3 (Egr3), which is a key target gene activated by SRF in the NRG1-ErbB pathway. In these cells, transcription of Egr3 is induced by NRG1 consistent with wild-type cells. These results suggest that transcriptional induction of Egr3 by NRG1 occurs independently of the N- and C- terminal domains of SRF and the MADS box is sufficient for induction. Future experiments will further investigate the MADS box and its interactions with known cofactors in the development of muscle spindles.

Acknowledgements

I would like to thank Dr. Larry Fromm for his support and guidance during this project and my undergraduate career. I am forever grateful for the laboratory experience and knowledge I gained from him throughout my time in the Fromm lab.

I would like to thank my daughter Quinne, for serving as my inspiration, and mother Stacy, whose constant support allowed me to pursue undergraduate research and excel in my college career.

Process Analysis

Diseases which target muscles and their nerve connections are not completely understood, which is reflected in the number of therapeutics available for them. According to Center of Disease Control statistics, hundreds of young males aged 5 – 24 are diagnosed with muscular dystrophy every year. Sensory neuropathies caused by infection, inheritable disease, trauma, or diabetes are estimated to affect 20 million people in the United States alone. While symptoms can be managed, there are currently no cures for muscular dystrophy or sensory neuropathies. Serum response factor (SRF) is a protein which has been shown to play a role in the formation of muscle spindles, which are muscle fibers innervated with nerves. In this study, we focus on how SRF is targeted in a specific pathway which is essential for muscle spindle development. Previous studies have shown promise for using SRF as a therapeutic target in disease, and studying how it functions in muscle spindle development gets us closer to applying this knowledge to treatment of muscular disorders and sensory neuropathies.

The MADS box of serum response factor is sufficient for induction of muscle spindle-specifying gene Egr3 by neuregulin is a significant study which has created a useful biotechnology tool in the form of a cell line expressing mutated forms of our protein of interest, SRF. During this project, I utilized many molecular biology techniques including recombinant DNA creation, polymerase chain reaction (PCR), cell culture techniques, and protein isolation and analysis to work towards our goal of assessing the function of SRF in a muscle spindle development pathway. CRISPR/Cas9 is an emerging genome editing technology which comprised a major premise of my experiments. This work is novel because the function of different domains of SRF in this pathway have not been previously characterized.

This work has not only prepared me for a career in research, but it has contributed to the Fromm laboratory and the scientific community. The outcome of this project, a mutant cell line, will benefit the Fromm laboratory in possible future experiments where the role of SRF in muscle spindle formation will be investigated further. In addition, we have demonstrated efficiency of an experimental approach for genome editing using CRISPR and isolating cells which express a particular genotype. The goal of our research was to learn more about how SRF functions in muscle spindle development and our results benefit the scientific community by providing insight on the molecular interactions that occur in the induction of muscle spindles. In the future, SRF may be a novel therapeutic target in diseases of muscular disorders and sensory neuropathies.

Introduction

Muscle spindles are an essential part of the neuro-muscular system because they act as sensory receptors of muscle and limb position that communicate with the nervous system via afferent nerve fibers and sense movement. Spindles consist of specialized muscle fibers, known as intrafusal muscle fibers and axons of the sensory neurons that innervate them (1). Muscle fibers require inductive cell signaling interactions with sensory axons to become intrafusal muscle fibers (2). The neuregulin-1 (NRG1)-ErbB pathway is a cell signaling pathway known to induce specification of intrafusal muscle fibers. In this pathway, NRG1 is a signaling protein released by sensory axons, which then activates its receptors, known as ErbBs, in muscle cells that are contacted. Early growth response 3 (Egr3) is an essential protein in this pathway which acts downstream of ErbBs and functions as a transcription factor that activates the target genes that form intrafusal muscle fibers. Egr3 has been shown to be activated by NRG1-ErbB signaling at the level of its own transcription through transcription factors serum response factor (SRF) and cAMP response element binding (CREB) (3).

Current understanding of how SRF is activated by signaling pathways is that it occurs through two families of signal-regulated coactivators, myocardin-related transcription factors (MRTFs) and ternary complex factors (TCFs). These coactivators are known to interact with a domain within SRF known as the MADS box, which also functions as the DNA binding domain (4, 5). SRF recognizes and binds to specific recognition elements in regulatory regions of particular genes, and in conjunction with its interacting coactivators, induces the transcription of these target genes. Previous evidence suggests that SRF may be regulated by other means not involving MRTFs or TCFs in the NRG1-ErbB pathway in muscle cells (6). These other mechanisms potentially could be through SRF phosphorylation or through its interaction with other coactivators.

To study the function of SRF, the sequence can be divided into three general domains: an N-terminal region, a central DNA binding region (MADS box), and a C-terminal region. Both the N-terminal region and the C-terminal region are not well-characterized, but features such as phosphorylation sites suggest they could function in SRF activation. The C-terminal region has been shown to exhibit transcriptional activation function in transcriptional reporter assays in transfected cells (7, 8). In this study, both the C-terminal and N-terminal domains of SRF were deleted in cultured muscle cells to assess the role of these domains of SRF in NRG1 signaling, and more specifically transcriptional induction of Egr3 (**Figure 2**).

The experimental system being used in the current studies utilizes C2C12 cells, which is an established muscle cell line originating from mouse skeletal muscles that are used to study muscle developmental processes. These cells can be propagated as myoblasts and differentiated to form multinucleate myotubes. C2C12 cells have been used in previous experiments studying the NRG1-ErbB pathway (3, 6). The C2C12 cell culture system we are using involves stimulating the cells with soluble NRG1, which will start the NRG1-ErbB signaling cascade when NRG1 binds to ErbB receptors. Because Egr3 acts downstream of SRF, assessing Egr3 level will be used to analyze transcriptional response in the pathway and determine whether

mutant SRF affects signaling. We hope that stimulation of C2C12 myotubes with NRG1 models signaling from afferent nerve fibers in developing intrafusal muscle fibers.

In the current studies, DNA coding for SRF amino acids 88-265 was placed in a retroviral plasmid vector using PCR and DNA cloning. The resulting SRF-88-265-expressing retrovirus was transduced into C2C12 muscle cells, and endogenous SRF was subsequently inactivated using CRISPR/Cas9 in a pool of cells. Single cell cloning was used to isolate cells which had a homozygous frameshift mutation in endogenous SRF, which completely inactivated its function. The cells were differentiated in culture media and stimulated with NRG1. Real-time PCR (qPCR) was used to determine transcriptional activation by comparing *Egr3* levels in NRG1-stimulated and unstimulated cells.

Studying SRF and its function in NRG1 signaling is important in public health because understanding how muscle spindles are innervated can provide useful therapeutic targets in diseases where nerves and their connections are destroyed, such as sensory neuropathies, and muscular disorders. SRF has shown promise in being a therapeutic target (9), and investigating its role in NRG1 signaling may premise a novel therapeutic for these diseases, where there are currently limited options for effective treatments.

Methods

Creation of recombinant DNA

A plasmid containing human SRF (pCGN-SRF) (8) was linearized with BamHI. Linearized pCGN-SRF was added to PCR containing PrimeSTAR GXL DNA polymerase (Takara R050A). The forward primer contained a BamHI site, coding sequences for an HA epitope tag, and a portion of the SRF gene beginning at amino acid 88. The reverse primer contained a portion of the SRF gene ending at amino acid 265, followed by a stop codon and an XhoI site. PCR conditions were as follows: initial denaturation at 98°C for 5 minutes (98°C for 10 seconds, 55°C for 15 seconds, 68°C for 50 seconds X 28 cycles). PCR sample was purified with a gel extraction kit (Sigma NA1111) and restriction digested with BamHI and Xho. Digested PCR fragment containing and HA tag and SRF amino acids 88-265 was isolated by gel extraction. The fragment was ligated into BamHI-XhoI digested pENTR11 (Invitrogen) using T4 DNA ligase. This ligation was then transformed into DH5 α bacteria and selected on kanamycin plates. Plasmid DNA from bacteria was isolated using Mini-Prep kit (Sigma PLN70) and confirmed by BamHI-XhoI digestion and DNA sequencing. SRF 88-265 was then transferred from pENTR11 to pQXIB using LR clonase.

Cell culture

C2C12 myoblasts were propagated using growth media (Dulbecco's Modified Eagle Medium (DMEM) (Corning Scientific 15-013-CM), 20% bovine growth serum (GE Healthcare Life Sciences SH30541), 4mM glutamine, 50 ug/ml Gentamycin (Gibco 15750-060)). Differentiation to myotubes was induced by propagating cells to confluence and replacing media with differentiation media (DMEM, 4% horse serum (GE Healthcare Life Sciences SH30074), 4mM glutamine, 50 ug/ml Gentamycin) was added. Cells were incubated at 37°C and 10% CO₂.

Retroviral transduction

pQXIB SRF 88-265 was transfected into Bosc23 retroviral packaging line cells (10) using Lipofectamine. Bosc23 cells were seeded at 2.2×10^6 cells/35mm plate using DMEM, 10% bovine growth serum, 4mM glutamine, 50 ug/ml Gentamycin. The next day, media was removed and 3.3 ug of DNA and 6.6 uL Lipofectamine (Invitrogen 11668) were diluted in 500 uL Opti-MEM Reduced Serum Media (Gibco 31985070) and added to cells. Cells were propagated for two days. The media, which contained retroviral particles, was harvested and 2 ml was added to C2C12 myoblasts, seeded the day before at 6×10^4 cells on a 35 mm dish. 8ug/ml polybrene was also added to the cells. Transduced C2C12 cells were propagated and stably transduced cells were selected using Blasticidin (Invitrogen) treatment (8 ug/ml) for 4-6 days. The cells were then subjected to adenoviral transduction.

Adenoviral transduction

C2C12 cells that had been stably transduced with SRF 88-265 were seeded at 6×10^4 cells per 35mm plate. The next day, recombinant adenovirus containing guide RNA targeting nucleotide 228 of endogenous SRF and recombinant adenovirus containing Cas9 were added to the cells. Cells were propagated for one week in media containing blasticidin, splitting cells as needed to avoid crowding. Cells reached confluency and growth media was replaced with differentiation media. Cells were re-fed with differentiation media 2 days after. 2 days after the re-feeding, a cell lysate was made using RIPA lysis buffer and protease inhibitors.

Dilution cloning

A 96-well plate was prepared by adding 100 μ L of growth media to each well. 1×10^3 of retroviral/adenoviral transduced cells in 100 μ L was placed into well A1. Two-fold serial dilutions were performed down column 1 and then 100 μ L of growth media was placed in each well of column 1, giving a final volume of 200 μ L. Two-fold serial dilutions were performed across all rows, starting with column 1. WT C2C12 were diluted to 2.5×10^3 cells/ml in 14 ml of suspension. 100 μ L of this suspension was added to each well of the 96-well plate to help transduced mutant SRF cells grow. Cells were propagated for two days and then treated with Blasticidin (8 μ g/ml) to kill WT cells. Selection continued for three days. Wells in which clonal populations appear to have risen from a single cell were isolated and expanded to larger plates gradually. These clones were differentiated and lysates were harvested for analysis.

SDS-PAGE/Western blot

Cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl, (pH 8.0), 150mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.4mM EDTA) with 1% protease inhibitor cocktail (Sigma P8340). Lysates were treated with SDS sample buffer (200 mM Tris-HCl, pH6.8, 400 mM DTT, 8 % SDS, 0.4% Bromophenol Blue, 40% glycerol) and incubated at 95°C to induce denaturation. Samples were run on a 10% polyacrylamide gel and transferred to a PVDF membrane (GE Healthcare Life Sciences 10600029) using the Hoefer Semi-Dry Transfer apparatus. The membrane was blocked with 5% milk in TBST (137 mM Sodium Chloride, 20 mM Tris-HCl, pH 7.6, 0.1% Tween-20) for an hour to prevent nonspecific binding of antibody. The blot was incubated in either 1:1000 dilution of SRF primary antibody (Cell Signaling Technology D71A9) or HA-tag primary antibody (Cell Signaling Technology C29F4) in blocking buffer overnight. The membrane was incubated in 1:2000 dilution of anti-rabbit IgG secondary antibody (Cell Signaling Technology 7074) for an hour and subjected to peroxidase chemo-luminescence reagents (Thermofisher Scientific 1859698 & 1859701) before imaging with the Kodak Image Station 4000.

qPCR

Differentiated myotubes in 35mm dishes were serum-starved for two hours by replacing differentiation media with DMEM. Plates were either treated with neuregulin1 (HRG β 1, R&D

Systems, 100 ng/ml) or left untreated for approximately 2 hours. RNA was extracted using RNA STAT-60 (Tel-Test CS502). Reverse transcriptase (RT) PCR using Affinity-Script cDNA Synthesis Kit (Stratagene 200436) created complementary DNA (cDNA) from 3 ug extracted RNA and random primers. cDNA was used in real-time (qPCR) using Brilliant SYBR Green QPCR Kit (Stratagene 600828) with Egr3 primers GCCCATTACAATCAGATGGCT (forward), GCTCGAATAAGAGAGTTCCGGA (reverse) or with GABP α primers to normalize expression.

Results and Discussion

The functions of the N- and C-terminal domains of SRF are unknown, but features such as phosphorylation sites and transcriptional activation function suggest that these domains could be involved in activation of SRF in the NRG1-ErbB pathway. If these domains are required for the signaling cascade, it would suggest that SRF functions independent of known signaling-regulated cofactors. To study the effect of the N- and C-terminal domains of SRF, a mutant form lacking these domains (containing amino acids 88-265) was introduced into C2C12 muscle cells and the endogenous form of SRF was inactivated using CRISPR/Cas9. These cells were then stimulated with NRG1 to assess the efficiency of the NRG1-ErbB pathway in cells lacking the N- and C-terminal domains of SRF. Level of Egr3 expression is indicative of the transcriptional response to NRG1 that occurs through SRF because Egr3 transcription in response to NRG1 requires SRF (3) (**Figure 1**).

The N- and C-terminal domains were studied by deleting the coding sequences within SRF and assessing the effects. SRF 88-265 was created by removing the N- and C- termini of SRF using PCR-based mutagenesis (**Figure 2**). Using retroviral transduction, SRF 88-265 was transduced into C2C12 myoblasts. C2C12 cells expressing SRF 88-265 were subjected to CRISPR, in which adenoviral transduction was used to introduce guide RNA (gRNA) and Cas9 to target endogenous SRF for inactivation. Because the gRNA targets a sequence in the N-terminus, SRF 88-265 was not affected by CRISPR. SRF expression was analyzed by Western blot analysis. Expression of endogenous SRF was reduced in a pool of cells using CRISPR/Cas9 (**Figure 3**). Wild-type C2C12, which contain only normal SRF (508 amino acids), cells transduced with SRF amino acids 88-508, and cells transduced with SRF 88-265 that had not been subject to CRISPR/Cas9 were controls, which show similar expression level of endogenous SRF. SRF 88-265 transduced with CRISPR/Cas9 shows a lower expression for endogenous SRF, indicating that endogenous SRF is partially inactivated.

The gRNA was designed to target the SRF gene to be cut by Cas9. After Cas9 cuts at the targeted site within the SRF gene, non-homologous end joining repairs the DNA at the double strand break. This type of repair typically creates short deletions or occasionally insertions (11, 12) Ideally, the repair would result in a frameshift mutation, disrupting the translation of SRF downstream of the gRNA target sequence. Because we targeted SRF in the N-terminus, a frameshift mutation would result in no translation of the MADS box and the C-terminal domain, which would completely inactivate the function of endogenous SRF. However, sometimes the DNA repair results in small in-frame deletions due to randomness of non-homologous end joining. In this case, endogenous SRF with a small internal deletion would be generated and might still be functional. The cell pool created presumably contains some cells that are still expressing endogenous SRF with small in-frame deletions, some which are heterozygous for a frameshift mutation, and some which are a homozygous for a frameshift mutation. A homozygous frameshift mutation would completely inactivate endogenous SRF, allowing us to assess the effect of SRF 88-265 on NRG1 signaling without interference of endogenous SRF.

To isolate cell clones from this pool which contained a homozygous frameshift mutation of endogenous SRF, dilution cloning was used. Multiple clonal populations originating from a single cell were isolated and genotypes were assessed using Western blot. Clonal population G2 shows no expression for endogenous SRF, indicating homozygous inactivation of the endogenous SRF gene, while expressing mutant SRF 88-265 (**Figure 4**). Seven other clonal populations that were analyzed show varying expression levels of endogenous SRF. Not all clonal populations contained cells which could fuse, which is likely due to the procedure of dilution cloning, which can cause cellular stress and can isolate cells that favor fibroblastic instead of myoblastic characteristics.

In clone G2, endogenous SRF expression was replaced with mutant SRF 88-265, which lacked the N- and C-terminal domains. These cells were propagated to confluence and switched to differentiation media, inducing myotube differentiation. It is known that SRF is essential for muscle cell differentiation, which is the formation of elongated, multinucleate myotubes from myoblasts (**13**). Clone G2 was able to form myotubes even though it lacked the N- and C-terminal domains of SRF (**Figure 5**), which suggests that only the MADS box (amino acids 88-265) is necessary for myotube differentiation. Myogenesis and muscle spindle development presumably occur via different signaling pathways, so although the N- and C-terminal domains of SRF is not necessary for myotube formation, it could still have a role in transcriptional activation in NRG1 signaling.

To assess transcriptional activation within the NRG1-ErbB pathway, myotubes from clone G2 were treated with NRG1 (**Figure 1**). Stimulating with NRG1 models NRG1 input from sensory axons *in vivo*. The extent of Egr3 transcription in response to NRG1 was evaluated using real-time qPCR with a NRG1 treated and an untreated control. We found that Egr3 expression, both basal expression and transcriptional induction by NRG1, was consistent among wild-type C2C12 cells with full-length SRF and mutant C2C12 cells with truncated SRF 88-265 (**Figure 6**). This suggests that the N- and C-terminal domains do not function in transcriptional activation of Egr3 in NRG1 signaling, and therefore only the MADS box, which mediates DNA binding and interaction with known signal-regulated coactivators, functions in the pathway. Levels of Egr3 in both control and mutant cells were higher in samples treated with NRG1 than in untreated, which aligns with results seen in Herndon et al (**6**).

In these experiments, a C2C12 muscle cell line expressing truncated SRF 88-265 was created to assess if the N- and C-terminal domains of SRF function in the NRG1-ErbB pathway to transcriptionally activate Egr3, an essential protein for muscle spindle development. We found that the N- and C-terminal domains do not function in transcriptional activation of Egr3, which suggests that within SRF, the MADS box, which mediates DNA binding and interaction with known signal-regulated coactivators, is sufficient for NRG1 signaling. The limitations of our study include not having more control samples for examining Egr3 expression, such a mutant SRF with only N-terminus deleted, having only a single mutant cell line for analysis, and lack of repeat experimental trials. In addition, cultured C2C12 cells may not behave consistently with *in vivo* cells after NRG1 stimulation. Future experiments include further investigating the MADS

box to study if it can function within the NRG1 pathway independent of known signal-regulated coactivators MRTFs and TCFs. SRF is known to be required for induction of the muscle-spindle gene *Egr3* by NRG1 (3). Studying how SRF is activated has implications for medicine which include possible ways to target SRF activation for therapeutic use in diseases such as muscular disorders and sensory neuropathies. SRF has shown promise for being a therapeutic target in disease (9). By targeting SRF activation, effectors of the NRG1-ErbB pathway could be upregulated to increase the transcription of muscle spindle-specific genes, promoting formation of muscle fibers of muscle spindles. This would be a novel therapeutic for muscular diseases and sensory neuropathies, where there are currently limited options for effective treatments.

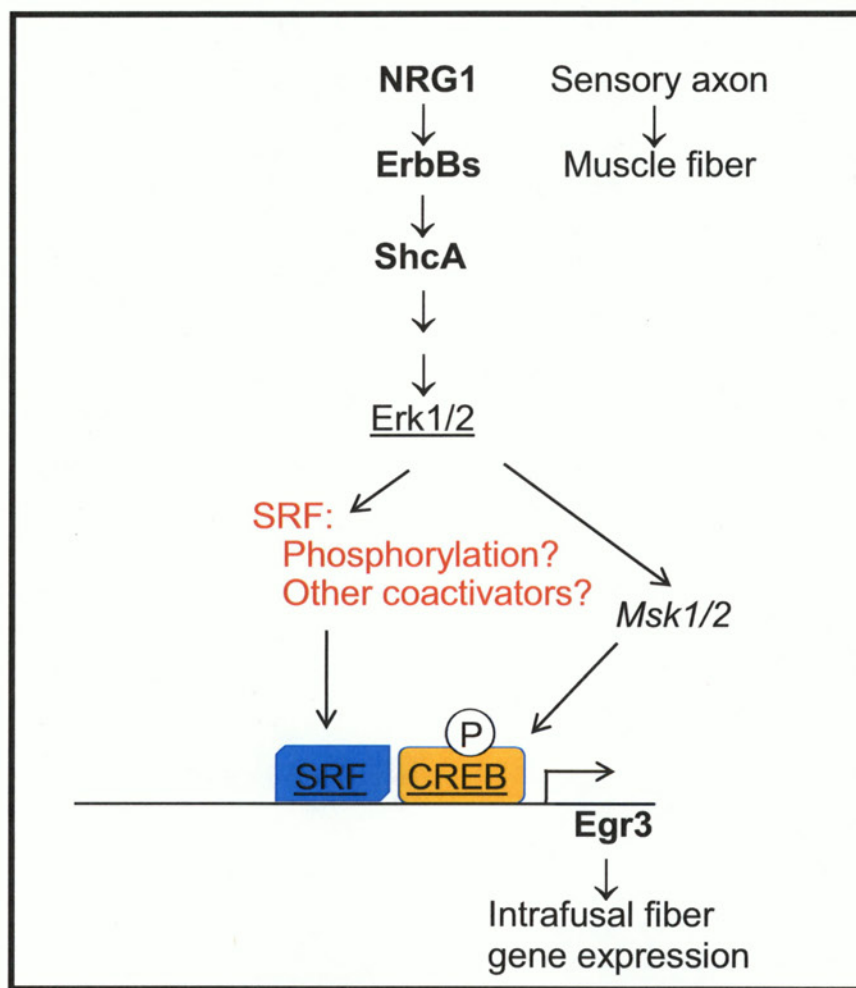


Figure 1. Model for the NRG1-ErbB signaling pathway that induces formation of muscle spindles. SRF has been shown to be a component of this pathway. Experiments here address how SRF might be targeted as part of the signaling response. Figure was modified from Dr. Larry Fromm.

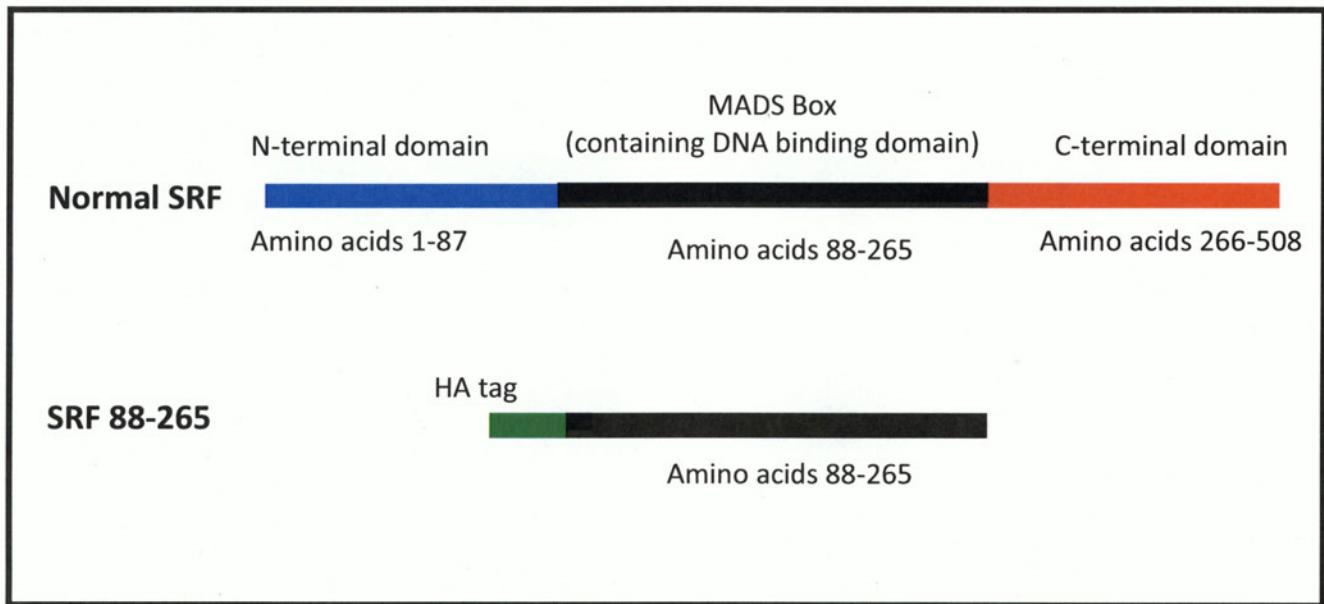


Figure 2. Mutant SRF 88-265 lacks N- and C-terminus. The truncated form of SRF was created by amplifying the nucleotides corresponding to amino acids 88-265 in PCR. Amino acids 88-265 include the DNA binding domain. An HA-tag was added N-terminal to amino acid 88 and allows for detection of mutant SRF via Western blot.

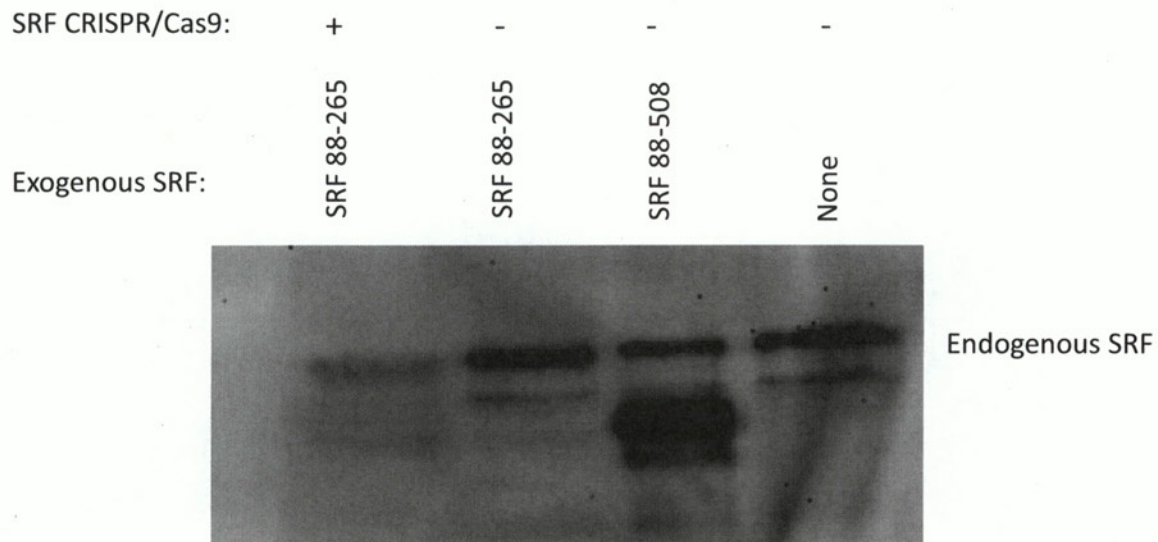
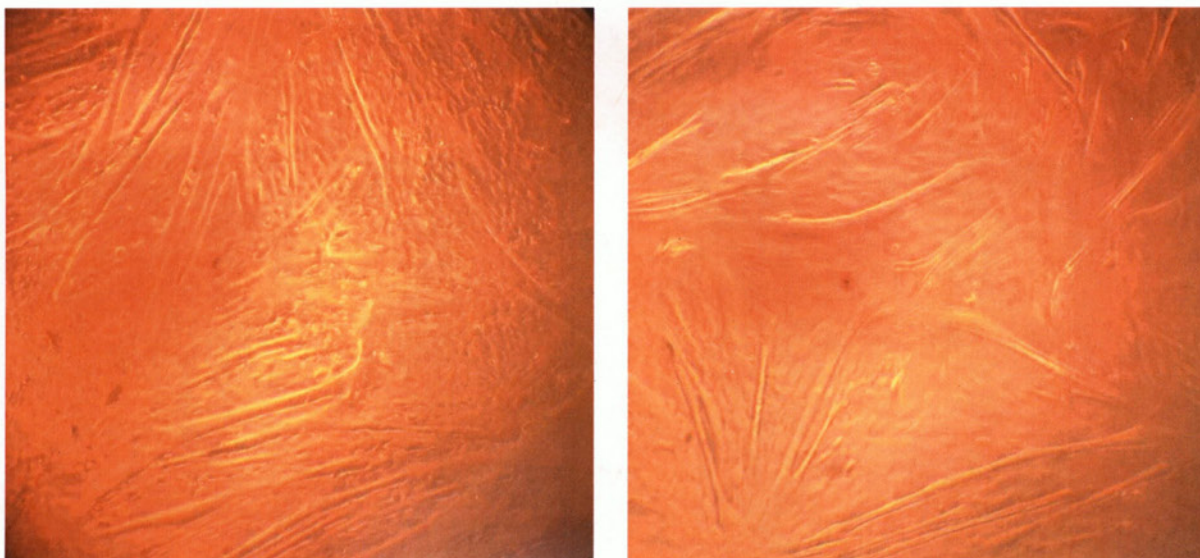


Figure 3. Generation of a pool of C2C12 cells that express truncated SRF 88-265 and that partially inactivate endogenous SRF. C2C12 cells were generated by retroviral transduction to express exogenous truncated forms of SRF that contain an HA epitope tag. Endogenous SRF was partially inactivated with CRISPR, in which SRF gRNA targeting the SRF gene corresponding to amino acid 76 and Cas9 were introduced by adenoviral transduction. Expression of endogenous SRF was assayed by Western blot using an antibody to SRF that recognizes an epitope that is C-terminal to amino acid 265. Residual endogenous SRF expression presumably reflects not all gRNA targeting events resulting in frameshift mutations. Blot was reprobed with HA antibody, which demonstrated expression of exogenous SRF (not shown, see figure 2B). Equal loading was confirmed by Coomassie staining.



Figure 4. Clonal population G2 has no expression of endogenous SRF while expressing truncated SRF 88-265. C2C12 cell populations from SRF 88-265/CRISPR/Cas9 pool were isolated using dilution cloning and assayed by Western blot. Expression of endogenous SRF was visualized by probing blot with SRF antibody that detects an epitope C-terminal of amino acid 265 (A). Sample G2 shows no expression of endogenous SRF, which suggests that all cells in the sample have a homozygous frameshift mutation in both alleles for the gene. Sample SRF 88-265 is a positive control showing full expression of endogenous SRF. Expression of truncated SRF 88-265 was visualized by re-probing blot with an antibody that recognizes the HA-tag present in the protein (B). Equal loading was confirmed using Coomassie staining (not shown).



C2C12

G2

Figure 5. C2C12 cells lacking the N- and C-terminal domains of SRF are able to form myotubes. Endogenous SRF was completely inactivated in clonal population G2 using CRISPR. G2 expresses truncated SRF with amino acids 88-265 (see figure 4). Lack of N- and C-terminal SRF did not affect the cell's ability to form myotubes, which are differentiated, multi-nucleate fibers.

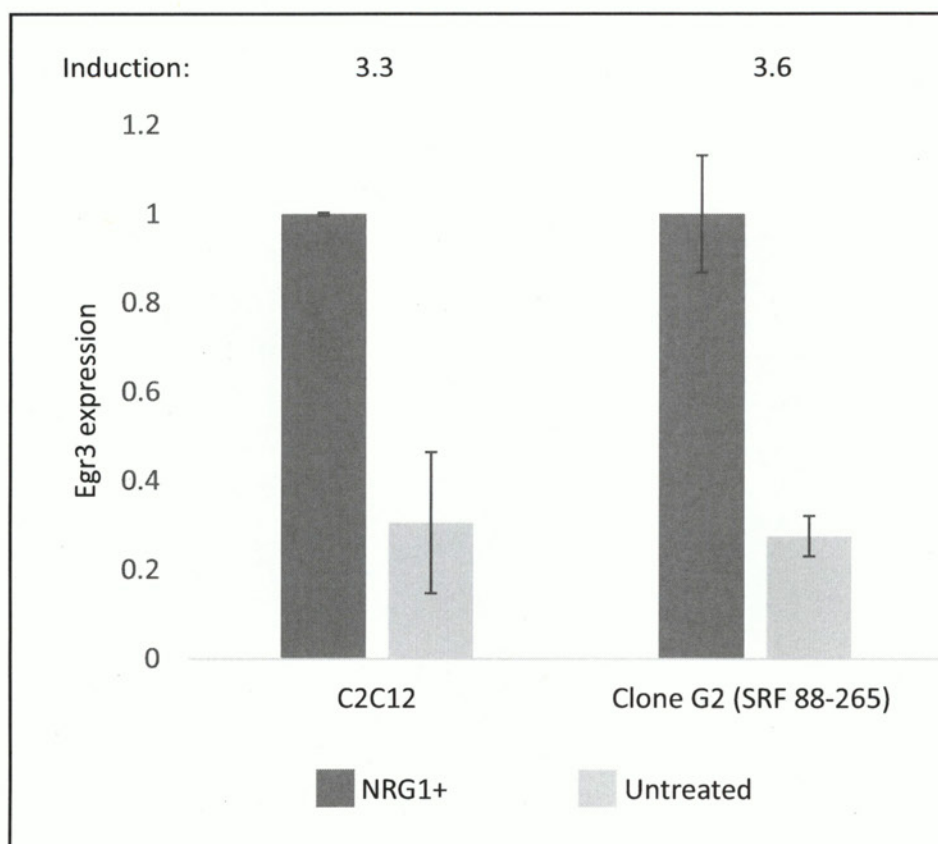


Figure 6. Egr3 expression in response to NRG1 was consistent among wild-type C2C12 and C2C12 with SRF 88-265. RNA was extracted from differentiated myotubes that had been stimulated with NRG1 or left untreated and subjected to reverse transcription. Complementary DNA was analyzed by real-time PCR using primers for Egr3. GABPa expression, which is not affected by NRG1, was used to normalize Egr3 expression values. Wild-type C2C12 cells express full-length SRF while SRF 88-265 express the mutant truncated form lacking N- and C-terminus.

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